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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02077219.0

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For the President of the European Patent Office

Le Président de l'Office européen des brevets
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R C van Dijk

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Anmelder/Applicant(s)/Demandeur(s):

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(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
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New chemical entities to modulate the activity of exchange proteins directly actrivated by camp (Epacs)

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(102)

NEW CHEMICAL ENTITIES TO MODULATE THE ACTIVITY OF EXCHANGE PROTEINS DIRECTLY ACTIVATED BY CAMP (EPACS)

Epacs are a family of guanine nucleotide exchange factors for small GTPases of the Ras family. These proteins are activated by direct binding of cAMP. By rational design we have identified and synthesized new chemical entities that specifically activate Epacs. The new chemical entities include cAMP analogues with a modified 2'-O-ribose group.

Introduction

cAMP, the first identified and well studied second messenger (1) , plays a role in a wide variety of cellular processes. Generally, it was assumed that the effects of cAMP are mediated by PKA, the ubiquitously expressed intracellular receptor for cAMP, although additional cAMP targets have been described, like the olfactory and pacemaker channels. Interestingly, recently we and others identified a family of Rap1 guanine nucleotide exchange factors directly activated by cAMP [Epac1 and Epac2 (also known as cAMP-GEFI and cAMP-GEFII)](2-4) . These widely-expressed proteins contain a cAMP binding pocket that is very similar to the cAMP binding pocket in the regulatory subunits of PKA, and cAMP is critically required for exchange activity of Epac1 and Epac2 towards the small GTPases Rap1 and Rap2.

Commonly used reagents to activate PKA, like forskolin, which activates adenylate cyclase, and 8-Br-cAMP, activate both the PKA- and the Epac mediated signalling pathways. To circumvent this problem we have searched for a specific stimulus to activate the Epac-Rap1 signalling pathway. Since Epac differs from PKA in the cAMP binding domain on at least one critical amino acid, we developed an Epac specific cAMP analogue, 8CPT-2Me-cAMP, that specifically

binds and activates Epac but not PKA in vitro. Using this analogue in vivo, we found that Rap1 is activated efficiently, whereas PKA-mediated responses are not induced. 8CPT-2Me-cAMP affected neither the activation nor the inactivation of ERK. Instead, we found cAMP-induced ERK activation to be critically dependent on PKA and Ras, whereas Rap1 activity was completely dispensable. The results clearly demonstrate and further strengthen the idea that cAMP-induced Rap1 activation and cAMP regulation of ERK are independent processes.

Results

A cAMP analogue specific for Epac Comparing the amino acid sequences of the cAMP binding domains of Epac with all other cNMP binding domains described in literature, including the cAMP domains of PKA, olfactory and pacemaker channels and the bacterial CAP protein, we noticed that the highly conserved glutamate that makes hydrogen bonding with the 2'-OH of the ribose group of cAMP (5) was absent the cAMP binding domain of Epac1 and in the high affinity cAMP binding domain-B of Epac 2 (Figure 1A). This suggested to us that this 2'-OH group, which is absolutely required for high affinity binding of cAMP to the cAMP binding domain of PKA, might not be required for efficient binding to and activation of Epac. We synthesized and tested a large number of compounds (synthesis by Biolog, modified after Katoaka et al 6 , one of which 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate, or 8CPT-2Me-cAMP (Figure 1B), was a very efficient activator of Epac1 in vitro (detailed biochemical analysis of 8CPT-2Me-cAMP will be described elsewhere). Half-maximal activation of Epac1 was observed at 2.2 µM 8CPT-2Me cAMP compared to 30 µM for cAMP (Figure 1C). Interestingly, 8CPT-2Me-cAMP binding to Epac1

results in a three-fold higher maximal activity than cAMP (Figure 1C), showing that 8CPT-2Me-cAMP is a much more potent allosteric regulator of Epac1 than cAMP. In contrast, the ability of 8CPT-2Me-cAMP to activate the type I and type II holoenzyme of PKA was greatly impaired compared to cAMP (Figure 1D). These in vitro results indicate that 8CPT-2Me-cAMP may also be a very potent compound to discriminate between the Epac and the PKA signalling pathways in vivo as well. Therefore, 8CPT-2Me-cAMP was tested in NIH3T3-A14-Epac1 cells for the activation of Epac, using Rap1 as a read-out, and of PKA, using phosphorylation of the common PKA substrate CREB 29 as a read-out. Importantly, whereas 8-Br-cAMP induced both the activation of Rap1 and the phosphorylation of CREB (Figure 1E, upper panel), 8CPT-2Me-cAMP induced the activation of Rap1 only. Concentration course experiments show that 8CPT-2Me-cAMP already activates Rap1 at a concentration of 10 µM (Figure 1E, lower panel), but even at a concentration of100 µM, it did not induce CREB phosphorylation. Taken together, we conclude that 8CPT-2Me-cAMP is a highly specific and efficient activator of Rap1 and a very useful tool to discriminate between the PKA mediated and the Epac-Rap mediated signalling pathways.

In addition we tested a large number of other new chemical entities for their effect on Epac and PKA (Table 1 and 2). Whereas 8CPT-2'O-Me was clearly one of the best specific activators of Epac, most other compounds tested also activated Epac

Experimental Procedures

Reagents

Antibodies against phosphorylated CREB (directed against phosphorylated Ser $_{133}$) were obtained from Cell Signalling and antibodies against K-Rev/Rap1 were obtained

from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The following inhibitors and stimuli were used at concentrationsindicated, unless stated otherwise: H-89 (10 µM), was obtained from Biomol Research Laboratories Inc. (Plymouth Meeting, PA, USA). Forskolin (40 µM) was obtained from ICN, 8-Br-cAMP (1 mM) and 8CPT-2Me-cAMP from Biolog Life Science Institute.

Cells, plasmids, transfections

NIH3T3-A14 cells stably expressing Epac1 were grown in DMEM with 10% fetal calf serum and 2 µg/ml, OVCAR3 cells were maintained in RPMI with 10% fetal calf serum.

In vitro activation of cAMP-dependent protein kinase Cyclic AMP dependent protein kinase I and II were reconstituted from isolated subunits and assayed for kinase activity using 70 mM kemptide as substrate as described previously (7).

In vitro measurements of Epac activity

In vitro GEF assays were performed as described 3,10. More specifically, 600 nM Rap1b loaded with the fluorescent nucleotide mantGDP was incubated in the presence of 100-fold excess GTP and in the absence or presence of 150 nM Epac1 DEP1. Increasing concentrations of cAMP or 8CPT-2ME-cAMP were added and single exponential curves were fit to the data (see Figure 1C two left panels) to calculate reaction rates. Buffer conditions were 50 mM Tris pH7.4, 150 mM NaCl, 5% glycerol, 5 mM DTE, 60 µM GTP. Reactions were carried out in 96-well plates and measured in a Cary Eclipse from Varian Inc. using the manufacturers software.

Rap1 activation assays

Rap activation assays were performed as described

previously (9-11). Briefly, cells were lysed in lysis buffer containing 10% glycerol, 1% Nonidet P-40, 50 mM Tris-Cl pH7.5, 200 mM NaCl, 2mM MgCl2, 1 µM leupeptin, 0.1 µM aprotinin, 5 mM NaF, 1 mM NaVo3. Lysates were clarified by centrifugation and incubated with GST-tagged RalGDS-RBD precoupled to glutathione beads to specifically pull down the GTP bound forms of Ras and Rap1, respectively. Samples were incubated for 1hr at 4°C while tumbling. Beads were washed four times in lysis buffer, remaining fluid was removed with an insulin syringe. Proteins were eluted with Laemmli sample buffer and analyzed by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and Western blotting. As a control, Ras and Rap1 levels in whole cell lysates were determined.

Legend

Figure 1. Identification of a cAMP analogue specific for Epac.

a. Alignment of the cAMP binding domains of PKA, Epac, and olfactory- and pacemaker channels.

b. Structure of 8CPT-2Me-cAMP.

c. *In vitro* activation of Epac1. Upper panels, Rap1 loaded with fluorescent Mant-GDP and in the presence of 100-fold excess GTP was incubated with Epac1 DEP in the presence of increasing concentrations of either cAMP (left) or 8CPT-2Me-cAMP (right). Lower panels, the reaction rates for cAMP and 8CPT-2Me-cAMP (left) and the corresponding IC50 and v_{max} values (right).

d. *In vitro* PKA activity of either type I holoenzyme (PKAI) or type II holoenzyme (PKAII) at increasing concentrations of either cAMP or 8CPT-2Me-cAMP.

e. 8CPT 2Me-cAMP activates Rap1 but not PKA in vivo. Upper panel, NIH3T3-A14-Epac1 cells were treated for

15 min with increasing concentrations of 8CPT-2Me-cAMP in duplo. Cells were lysed and equal amounts of cell lysate were incubated with precoupled GST-Ral GDSRBD and Rap1 was assayed by immunoblotting with a Rap1 antibody. Phosphorylation of CREB in corresponding cell lysates was analyzed using a phospho-specific CREB antibody. Lower panel, as a control, cells were treated with 8-Br-cAMP for 15 min, and cell lysates were analyzed for activation of Rap1 and phosphorylation of CREB.

Table 1: Structure of new chemical entities

Table 2: Effect new chemical entities on PKA activation and Epac actrivation in vitro

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CLAIMS

1. Chemicals for modulating the activity of exchange proteins directly activated by cAMP.

2. Chemicals as claimed in claim 1, selected from the group consisting of:

2'-Deoxyadenosine-3', 5'-cyclic monophosphate;

8-Bromo-2'-deoxy adenosine-3', 5'-cyclic monophosphate;

8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphate;

8-(4-Chloro-phenylthio)- N^6-phenyl-2'-deoxyadenosine-3', 5'-cyclic monophosphate;

2'-O-Methyladenosine -3', 5'-cyclic monophosphate;

8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphate;

8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate;

2'-O-Ethyladenosine -3', 5'-cyclic monophosphate;

2'-O-Propyladenosine -3', 5'-cyclic monophosphate;

2'-O-n-Butyladenosine -3', 5'-cyclic monophosphate;

2'-O-*iso*Butyladenosine -3', 5'-cyclic monophosphate;

8-Methylamino-2'-O-methyladenosine-3', 5'-cyclic monophosphate;

8-Methylthio-2'-O-methyladenosine-3', 5'-cyclic monophosphate;

8-(4-Fluoro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Methyl-cumarinyl-7-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(Naphtyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Phenylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Nitro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(2-Amino-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Benzylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-n-Hexylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Phenylethylamino-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Isopropylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(Benzimidazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(2-Hydroxy-ethylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Ethylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(2-Amino-ethylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(Pyridinyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(Benzothiazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Methyl-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(3-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Isopropyl-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(2,3,5,6-Tetrafluoro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Hydroxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(2,4-Dichloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-(4-Chloro-phenylthio)-2'-(N,N-dimethyl)-carbamoyl-adenosine-3',5'-cyclic monophosphate;

8-Methoxy-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Benzyloxy-2'-O-methyladenosine-3',5'-cyclic monophosphate;

8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphorothioate, Sp-isomer;

8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphorothioate, Sp-isomer;

8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorothioate, Rp-isomer;

8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorothioate, Sp-isomer;

8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer;

8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Sp-isomer;

8-(4-Chloro-phenylthio)-inosine-3', 5'-cyclic monophosphate;

8-Cyclohexylamino-2'-deoxyadenosine-3', 5'-cyclic monophosphate;

3. Synthesis of chemicals as claimed in any one of claims 1 and 2 by any suitable method.

4. Use of chemicals as claimed in any one of claims 1 and 2 for a biological purpose, including, but not exclusive, the treatment of cultured cells, animals or human beings.

5. Use of chemicals as claimed in any one of claims 1 and 2 for a biological purpose, including, but not exclusive, the treatment of cultured cells, animals or human beings to modulate the activity of exchange proteins

directly activated by cAMP.

Fig. 1A

PKARIa-A	F	G	E	L	A	L	I	Y	G	T	-	-	-	-	-	-	-	P	R	A	A	T	V	K
PKARIb-A	F	G	E	L	A	L	I	Y	G	T	-	-	-	-	-	-	-	P	R	A	A	T	V	K
PKARIa-B	F	G	E	I	A	L	L	M	N	R	-	-	-	-	-	-	-	P	R	A	A	T	V	V
PKARIb-B	F	G	E	I	A	L	L	L	N	R	-	-	-	-	-	-	-	P	R	A	A	T	V	V
PKARIIa-A	F	G	E	L	A	L	M	Y	N	T	-	-	-	-	-	-	-	P	R	A	A	T	I	V
PKARIIb-A	F	G	E	L	A	L	M	Y	N	T	-	-	-	-	-	-	-	P	R	A	A	T	I	T
PKARIIa-B	F	G	E	L	A	L	V	T	N	K	-	-	-	-	-	-	-	P	R	A	A	S	A	Y
PKARIIb-B	F	G	E	L	A	L	V	T	N	K	-	-	-	-	-	-	-	P	R	A	A	S	A	H
Epac1	F	G	Q	L	A	L	V	N	D	A	-	-	-	-	-	-	-	P	R	A	A	T	I	I
Epac2-B	F	G	K	L	A	L	V	N	D	A	-	-	-	-	-	-	-	P	R	A	A	S	I	V
D.M.Epac-B	F	G	K	L	A	L	I	N	D	A	-	-	-	-	-	-	-	P	R	A	A	T	I	V
Cel.Epac-B	F	G	K	L	A	L	V	N	D	L	-	-	-	-	-	-	-	P	R	A	A	T	I	V
Epac2-A	F	G	E	S	-	I	L	D	N	T	-	-	-	-	-	-	-	P	R	H	A	T	I	V
Olfactory	F	G	E	I	S	I	L	N	I	K	G	S	K	M	G	N	-	R	R	T	A	N	I	R
Pacemaker	F	G	E	M	V	H	L	Y	A	K	P	G	K	S	N	A	D	V	R	A	L	T	Y	C

Fig. 1B

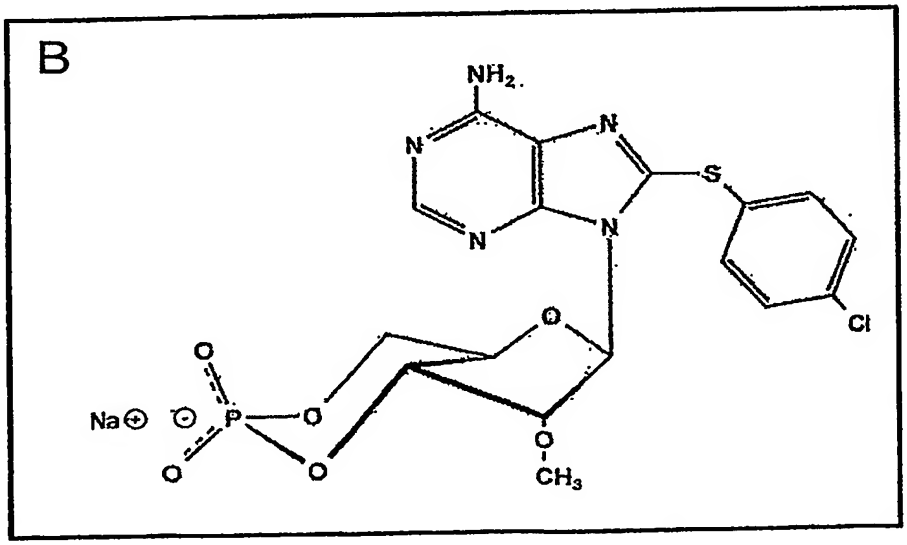
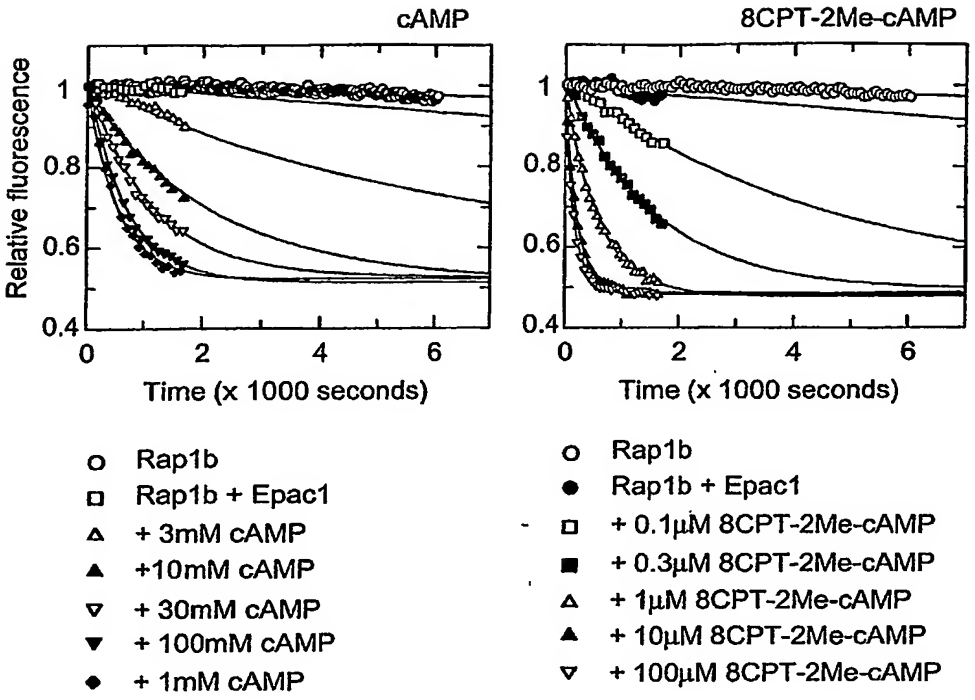
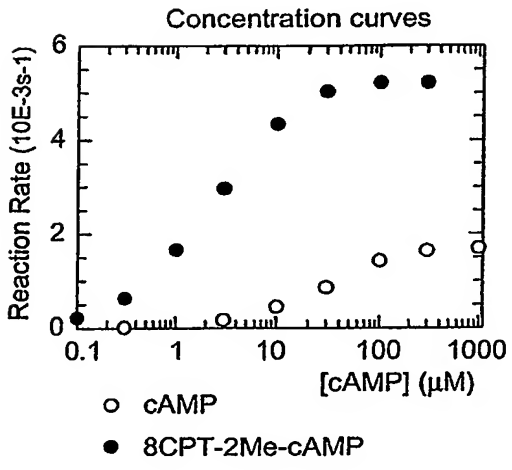


Fig. 1C





	cAMP	8CPT-2Me-cAMP
Ic50(µM)	30	2.2
Maximal activity (10E-3s-1)	1.72	5.2

Fig. 1D

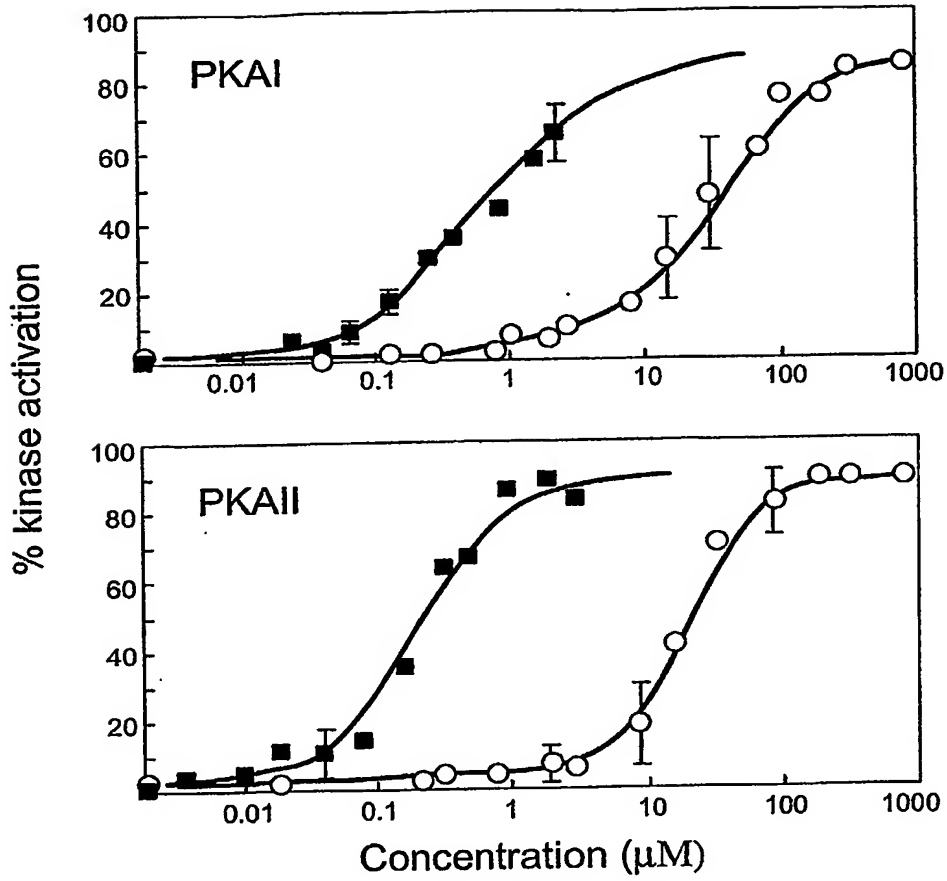
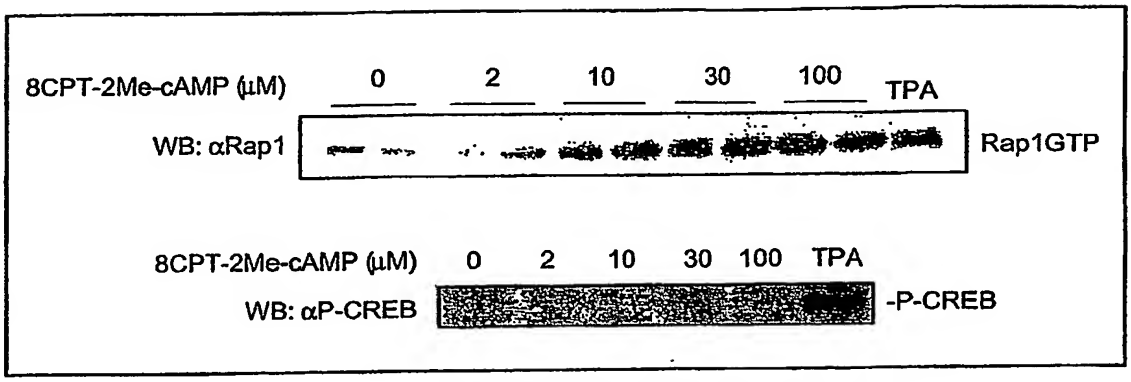

PKAI
PKAII
% kinase activation
100
80
60
40
20
0.01
0.1
1
10
100
1000
Concentration (µM)
cAMP
8CPT-2Me-cAMP

Fig. 1E


8CPT-2Me-cAMP (μM)
0
2
10
30
100
TPA
WB: αRap1
Rap1GTP
8CPT-2Me-cAMP (μM)
0
2
10
30
100
TPA
WB: αP-CREB
-P-CREB

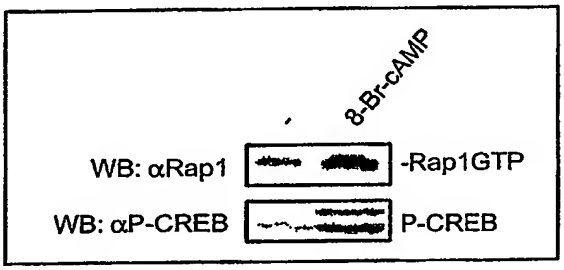
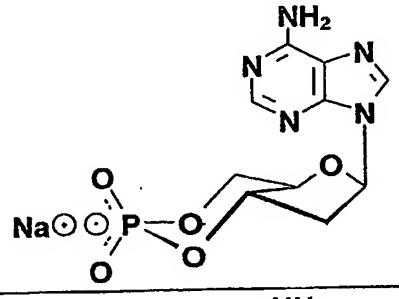
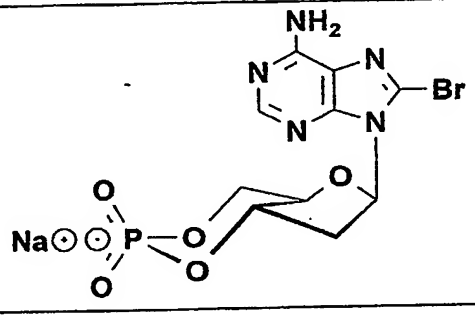
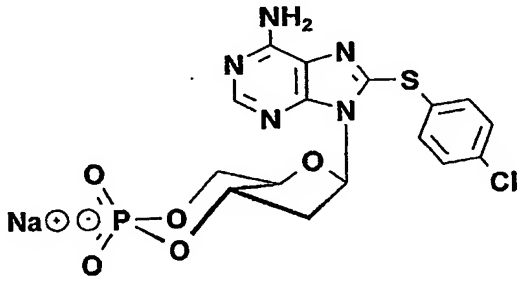
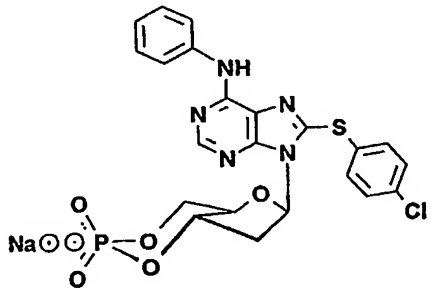
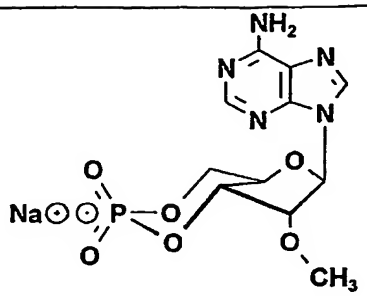
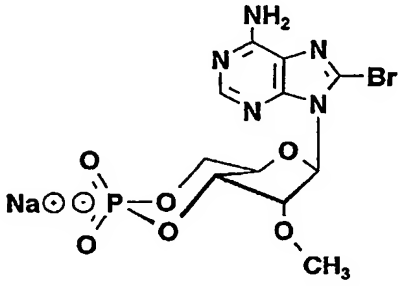
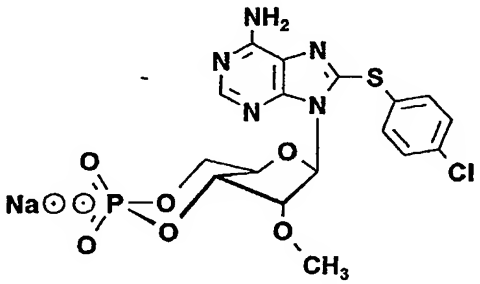
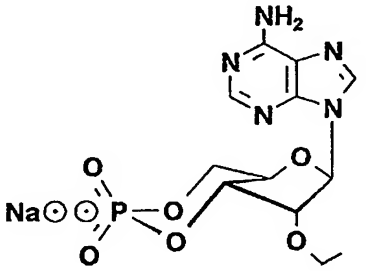
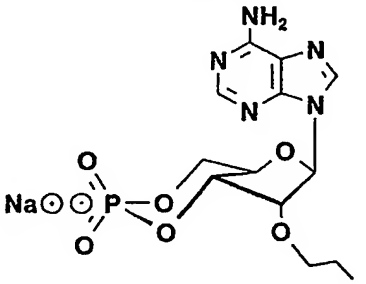
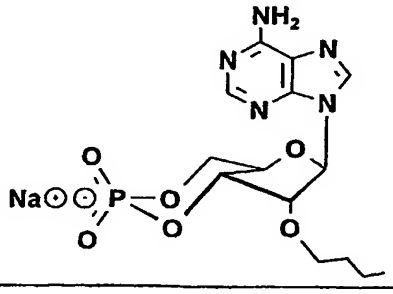
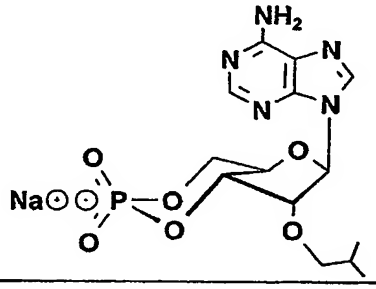
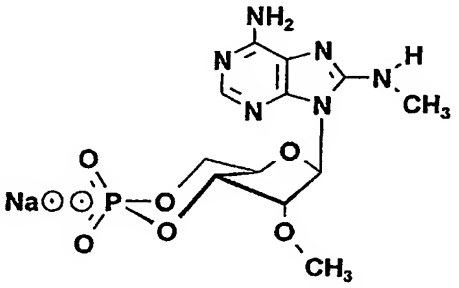
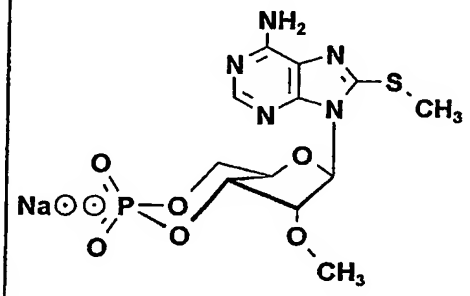
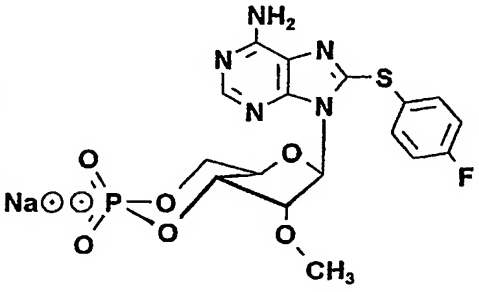
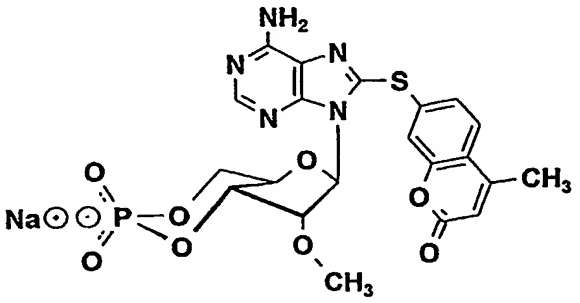
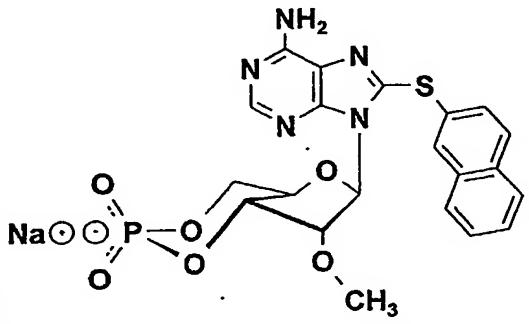
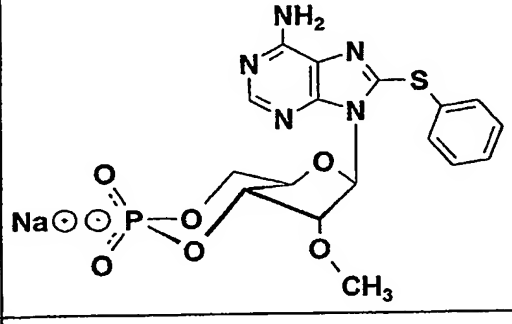
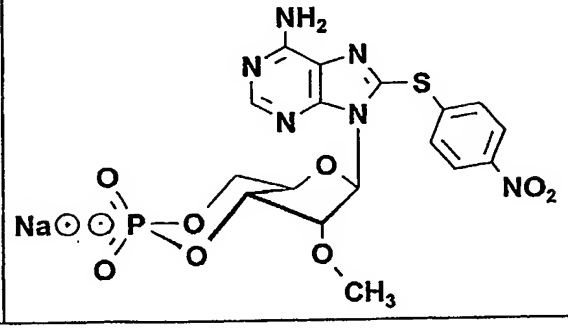

8-Br-cAMP
WB: αRap1
-Rap1GTP
WB: αP-CREB
P-CREB

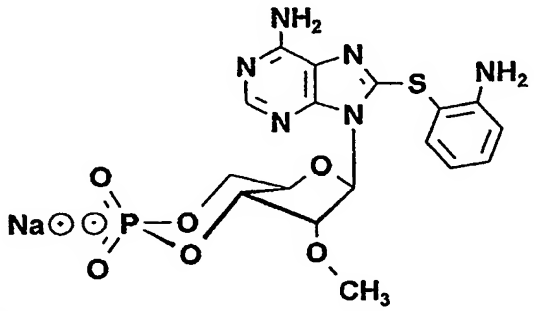
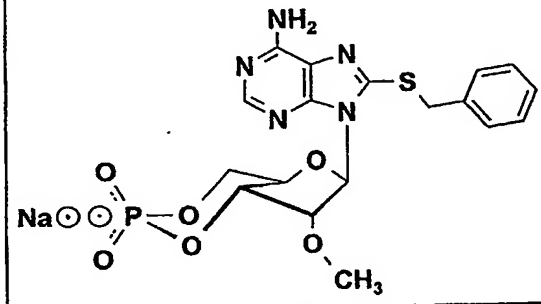
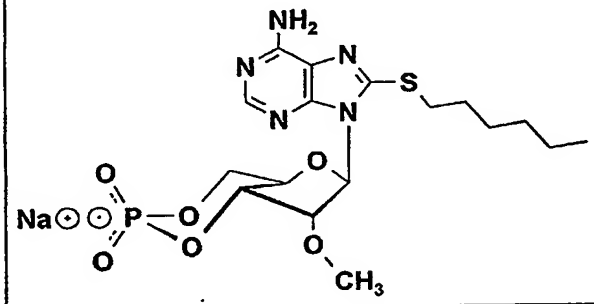
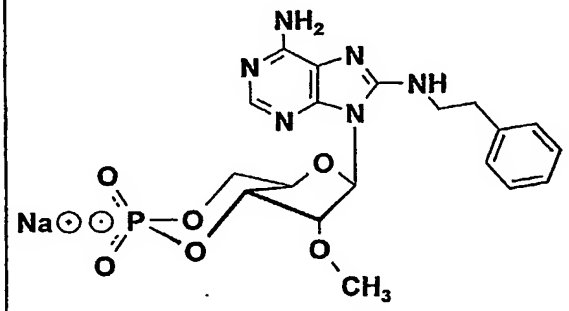
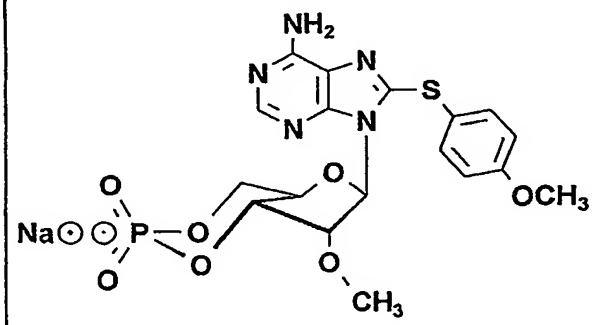
TABLE 1: EPAC-selective cyclic nucleotide analogs

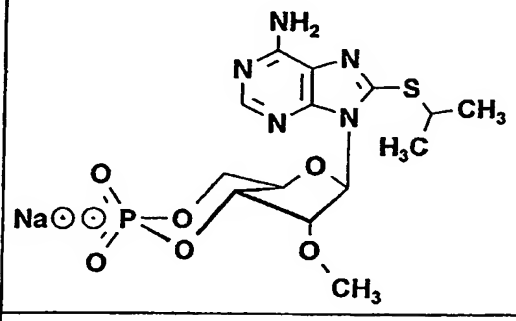
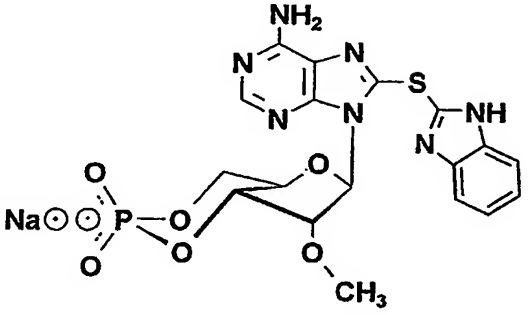
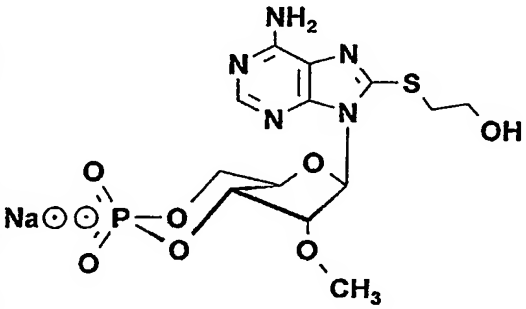
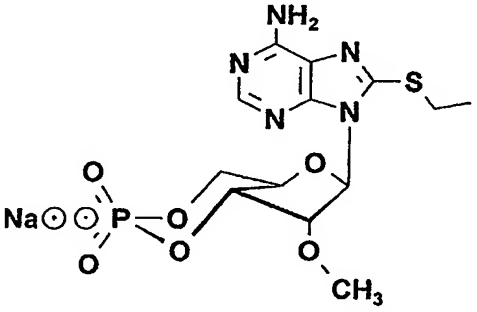
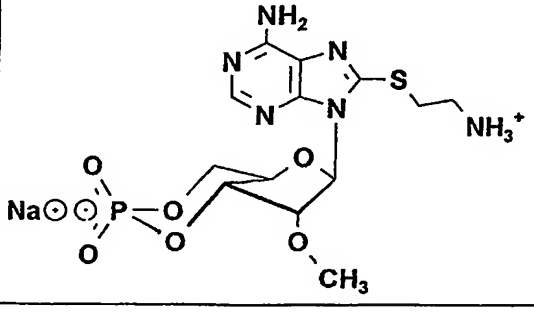
Compound name	Name	Chemical structure	activity
2'-dcAMP 2'-Deoxyadenosine-3', 5'-cyclic monophosphate	**I-001**		+
8-Br-2'-dcAMP 8-Bromo-2'-deoxy adenosine-3', 5'-cyclic monophosphate	**I-002**		+
8-*p*CPT-2'-dcAMP 8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphate	**I-003**		+
8-*p*CPT-6-Phe-2'-dcAMP 8-(4-Chloro-phenylthio)- N^6-phenyl-2'-deoxyadenosine-3', 5'-cyclic monophosphate	**I-004**		?

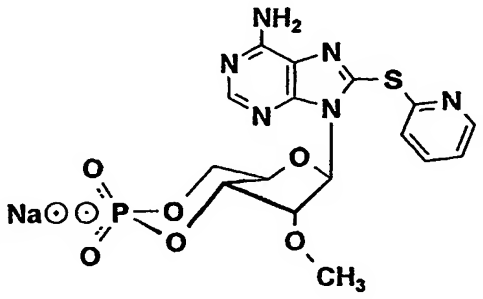
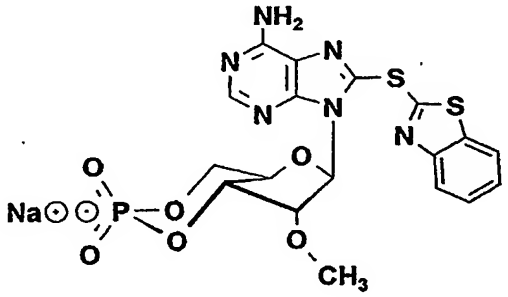
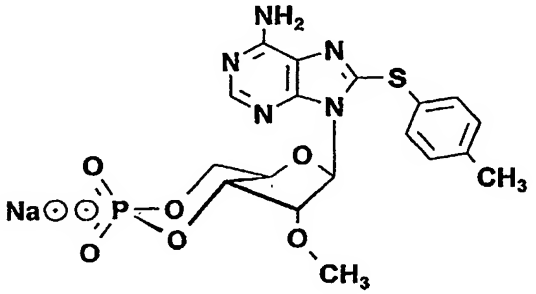
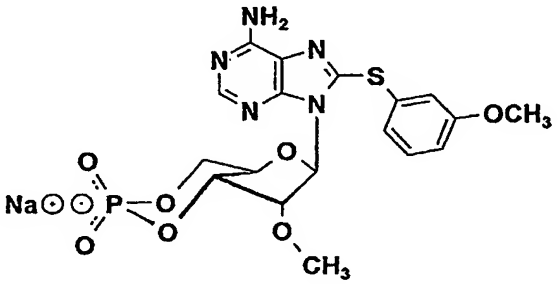
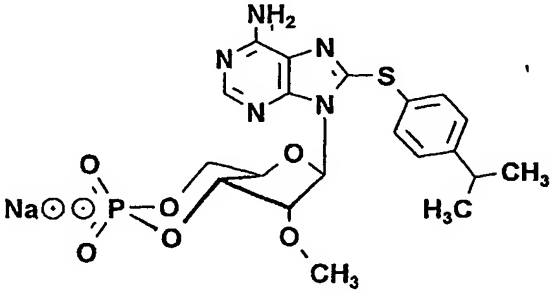
2'-O-Me-cAMP 2'-O-Methyladenosine-3', 5'-cyclic monophosphate	**I-005**		?
8-Br-2'-O-Me-cAMP 8-Bromo-2'-O-methyladenosine-3', 5'-cyclic monophosphate	**I-006**		++
8-*p*CPT-2'-O-Me-cAMP 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphate	**I-007**		+++
2'-O-Et-cAMP 2'-O-Ethyladenosine-3', 5'-cyclic monophosphate	**I-008**		+
2'-O-Pr-cAMP 2'-O-Propyladenosine-3', 5'-cyclic monophosphate	**I-009**		+

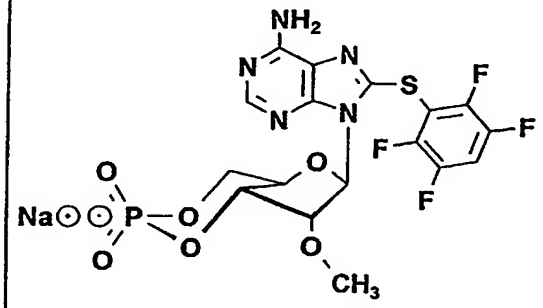
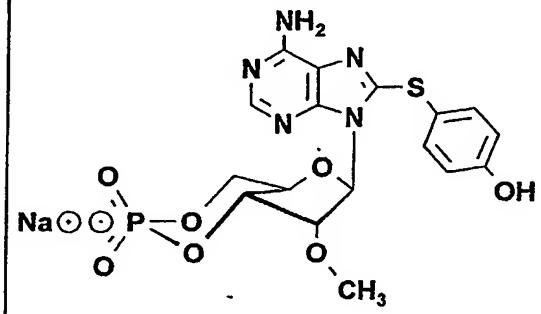
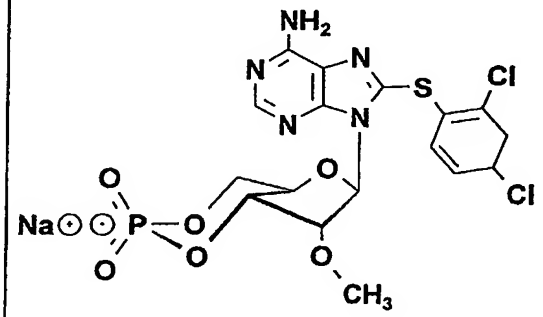
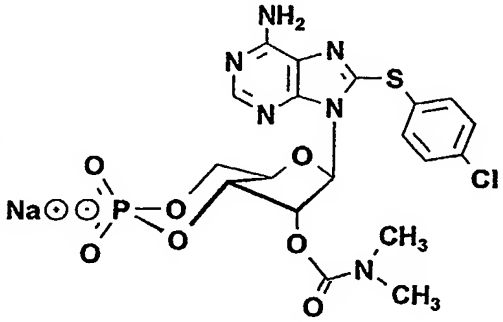
2'-O-Bu-cAMP 2'-O-n-Butyladenosine -3', 5'-cyclic monophosphate	**I-010**		+
2'-O-*i*Bu-cAMP 2'-O-*iso*Butyladenosine -3', 5'-cyclic monophosphate	**I-011**		-/+
8-MA-2'-O-Me-cAMP 8-Methylamino-2'-O-methyladenosine-3', 5'-cyclic monophosphate	**I-100**		-/+
8-MT-2'-O-Me-cAMP 8-Methylthio-2'-O-methyladenosine-3', 5'-cyclic monophosphate	**I-101**		+
8-*p*FPT-2'-O-Me-cAMP 8-(4-Fluoro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-102**		+++

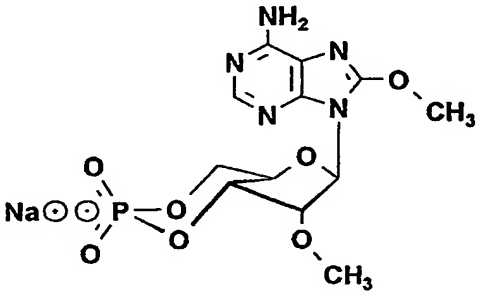
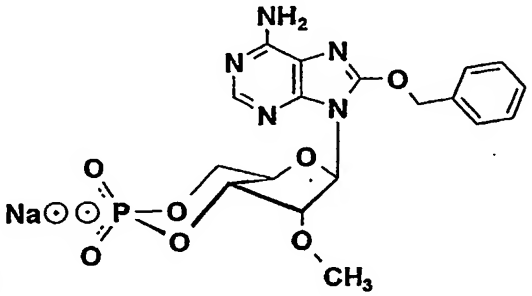
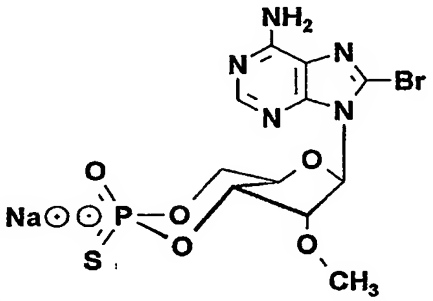
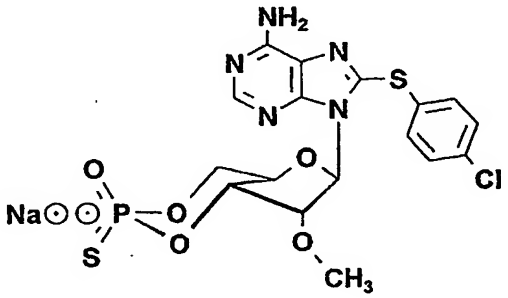
8-MCT-2'-O-Me-cAMP 8-(4-Methyl-cumarinyl-7-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-103**		-/+
8-NT-2'-O-Me-cAMP 8-(Naphtyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-104**		++
8-PT-2'-O-Me-cAMP 8-Phenylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-105**		++
8-*p*NPT-2'-O-Me-cAMP 8-(4-Nitro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-106**		++

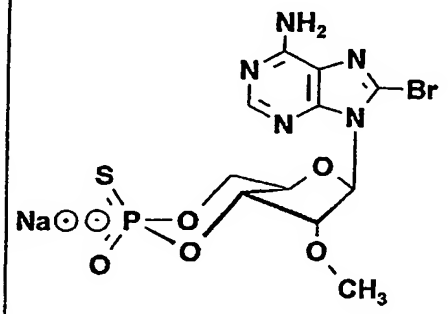
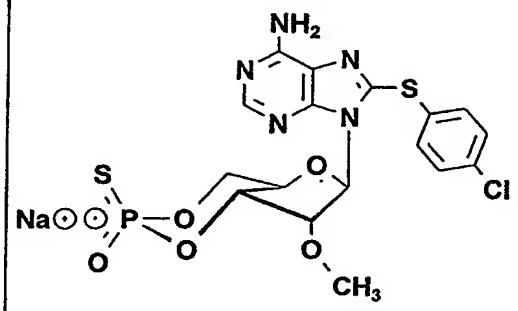
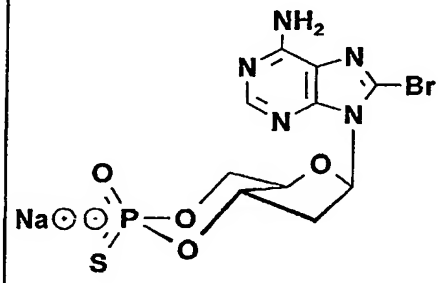
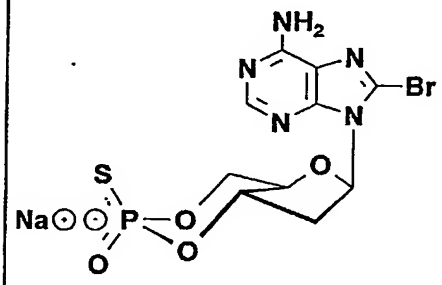
8-*o*APT-2'-O-Me-cAMP 8-(2-Amino-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-107**		+
8-BnT-2'-O-Me-cAMP 8-Benzylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-108**		+
8-HT-2'-O-Me-cAMP 8-n-Hexylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-109**		++
8-PhEA-2'-O-Me-cAMP 8-Phenylethylamino-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-111**		-/+
8-*p*MeOPT-2'-O-Me-cAMP 8-(4-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-112**		+++

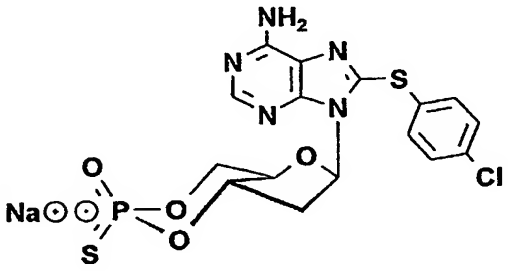
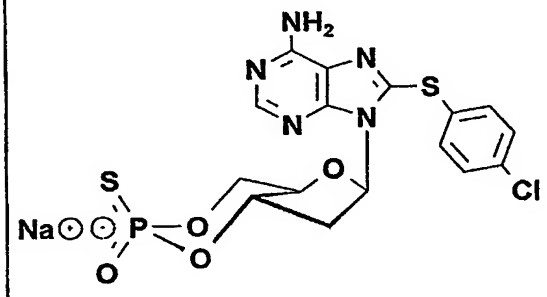
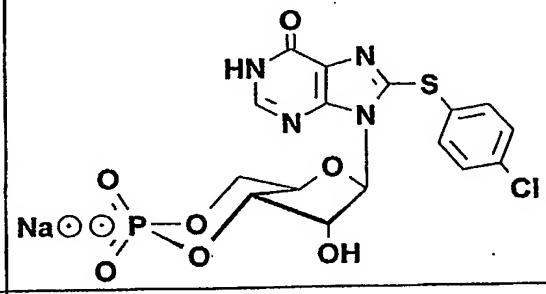
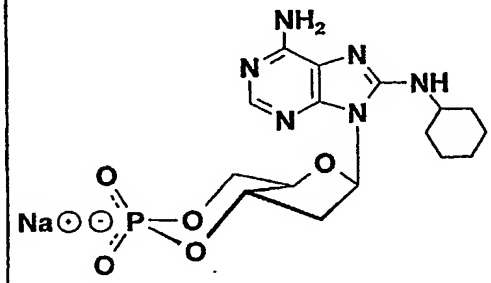
8-IPT-2'-O-Me-cAMP 8-Isopropylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-113**		+
8-BIT-2'-O-Me-cAMP 8-(Benzimidazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-115**		+
8-HET-2'-O-Me-cAMP 8-(2-Hydroxy-ethylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-116**		+
8-ET-2'-O-Me-cAMP 8-Ethylthio-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-117**		+
8-AET-2'-O-Me-cAMP 8-(2-Amino-ethylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-118**		-/+

8-PyT-2'-O-Me-cAMP 8-(Pyridinyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-119**		++
8-BTT-2'-O-Me-cAMP 8-(Benzothiazolyl-2-thio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-120**		+
8-pMPT-2'-O-Me-cAMP 8-(4-Methyl-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-121**		+++
8-*m*MeOPT-2'-O-Me-cAMP 8-(3-Methoxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-122**		+
8-*p*IPPT-2'-O-Me-cAMP 8-(4-Isopropyl-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-123**		++

8-TFPT-2'-O-Me-cAMP 8-(2,3,5,6-Tetrafluoro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-124**		+
8-*p*HPT-2'-O-Me-cAMP 8-(4-Hydroxy-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-125**		+++
8-DCPT-2'-O-Me-cAMP 8-(2,4-Dichloro-phenylthio)-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-126**		+
8-*p*CPT-2'-DMC-cAMP 8-(4-Chloro-phenylthio)-2'-(N,N-dimethyl)-carbamoyl-adenosine-3',5'-cyclic monophosphate	**I-127**		-

8-MeO-2'-O-Me-cAMP 8-Methoxy-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-131**		N.G.
8-BnO-2'-O-Me-cAMP 8-Benzyloxy-2'-O-methyladenosine-3',5'-cyclic monophosphate	**I-132**		N.G.
Rp-8-Br-2'-O-Me-cAMPS 8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphorothioate, Rp-isomer	**I-129**		-/+
Rp-8-*p*CPT-2'-O-Me-cAMPS 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer	**I-130**		-/+

Sp-8-Br-2'-O-Me-cAMPS 8-Bromo-2'-O-methyladenosine -3', 5'-cyclic monophosphorothioate, Sp-isomer	**I-133**		?
Sp-8-*p*CPT-2'-O-Me-cAMPS 8-(4-Chloro-phenylthio)-2'-O-methyladenosine-3', 5'-cyclic monophosphorothioate, Sp-isomer	**I-134**		?
Rp-8-Br-2'-dcAMPS 8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorothioate, Rp-isomer	**I-135**		?
Sp-8-Br-2'-dcAMPS 8-Bromo-2'-deoxyadenosine -3', 5'-cyclic monophosphorothioate, Sp-isomer	**I-136**		?

Rp-8-*p*CPT-2'-dcAMPS 8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Rp-isomer	**I-137**		?
Sp-8-*p*CPT-2'-dcAMPS 8-(4-Chloro-phenylthio)-2'-deoxyadenosine-3', 5'-cyclic monophosphorothioate, Sp-isomer	**I-138**		?
8-*p*CPT-cIMP 8-(4-Chloro-phenylthio)-inosine-3', 5'-cyclic monophosphate	**I-139**		?
8-cHA-2'-dcAMP 8-Cyclohexylamino-2'-deoxyadenosine-3', 5'-cyclic monophosphate	**I-140**		?

relative affinity	cAMP=1			relative activity of Epac *
	PKAI	PKAII	Epac	
I-001				+
I-002				+
I-003	0.001	0.001	0.2	+
I-004			0.5	ND
I-005	0.005	0.005	0.12	ND
I-006	0.0015	0.003	0.9	++
I-007	0.009	0.003	4.6	+++
I-008	0.0043		0.05	+
I-009	0.001		0.03	+
I-010	0.0008		0.03	+
I-011				±
I-100	0.0014	0.0001	<0.5	±
I-101	0.012	0.0006	1.3	+
I-102	0.02	0.0004	4.5	+++
I-103	0.03	0.0005	1.8	±
I-104	0.03	0.001	2.3	++
I-105	0.02	0.0005	3.6	++
I-106	0.02	0.0002	1.2	++
I-107	0.004	0.0001	1.3	+
I-108	0.04	0.0007	1.3	+
I-109	0.03	0.0008	1.2	++
I-111			0.08	±
I-112	0.025	0.0006	6.5	+++
I-113	0.028	0.0004	0.7	+
I-115			0.09	+
I-116			1.2	+
I-117			1.8	+
I-118			0.34	±
I-119			0.8	++
I-120			0.17	+
I-121			5.1	+++
I-122			2	+
I-123			4.8	++
I-124			4.7	+
I-125			6.4	+++
I-126			3.1	+
I-127			<0.002	-
I-129 (Rp-006)			0.05	±
I-130 (Rp-007)			0.06	±
I-131			0.36	+
I-132			0.18	+
I-133				+
I-134				+

I-135				+
I-136				+
I-137				+
I-138				+
I-139				+
I-140				+

* relative activity of Epac in vitro in the exchange reaction towards Rap
+++ activity comparible to 007
++ activity between 007 and cAMP
+ activity comparible to cAMP
± activity less than cAMP

TABLE 2